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Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway

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ABSTRACT **Jasmonic acid, synthesized from linolenic acid (the octadecanoid pathway), has been proposed to be part of a signal transduction pathway that mediates the induction of defensive genes in plants in response to oligouronide and polypeptide signals generated by insect and pathogen attacks. We report here that the induction of proteinase inhibitor accumulation in tomato leaves by plant-derived oligogalacturonides and fungal-derived chitosan oligosaccharides is severely reduced by two inhibitors (salicylic acid and diethyldithiocarbamic acid) of the octadecanoid pathway, supporting a role for the pathway in signaling by oligosaccharides. Jasmonic acid levels in leaves of tomato plants increased several fold within 2 hr after supplying the polypeptide systemin, oligogalacturonides, or chitosan to the plants through their cut stems, as expected if they utilize the octadecanoid pathway. The time course of jasmonic acid accumulation in tomato leaves in response to wounding was consistent with its proposed role in signaling proteinase inhibitor mRNA and protein synthesis. The cumulative evidence supports a model for the activation of defensive genes in plants in response to insect and pathogen attacks in which various elicitors generated at the attack sites activate the octadecanoid pathway via different recognition events to induce the expression of defensive genes in local and distal tissues of the plants.**

In leaves of tomato and potato plants, defensive genes are expressed both locally and distally in response to herbivore and pathogen attacks (1–4). Proteinase inhibitor genes can be activated by oligosaccharide fragments generated from both plant and fungal cell walls during pathogen attacks (5, 6) and by an 18-amino acid polypeptide called systemin that is thought to be released from plant cells damaged by chewing insects (7). The oligosaccharides are relatively immobile in plants and are considered to be localized signals, while the polypeptide is mobile and is the primary candidate as a systemic signal. The activation of defensive genes by wounding, oligouronides, and systemin has been proposed to occur via a lipid-derived pathway [the octadecanoid pathway (8)] in which linolenic acid is generated in receptor cells in response to the signals (9) and is converted to jasmonic acid (8), leading to the transcriptional activation of the defensive genes (9–11). Jasmonic acid synthesis from linolenic acid, a major fatty acid constituent of the membrane lipids of most plants, involves a series of steps analogous to the production of prostaglandins from arachidonic acid in animals, including oxidation and cyclization of the lipid precursors and, in the pathway to jasmonic acid, β-oxidations to shorten the fatty acid side chain (8). In animals, arachidonic acid is released in response to a variety of signals including polypeptide factors, whereas in plants, linolenic acid was hypothesized to be released from membranes in response to systemin or plant cell wall oligosaccharide fragments (9, 10).

A role for the octadecanoid pathway in signaling proteinase inhibitor genes by systemin (wounding) or by oligosaccharides (pathogen attacks) has been supported by several lines of evidence. Supplying young tomato plants with systemin or oligosaccharide fragments activates proteinase inhibitor and polyphenol oxidase genes (3, 5, 7). The topical application to tomato leaf surfaces of linolenic acid or of intermediates of the pathway from linolenic acid to jasmonic acid also activates defensive genes (9). Furthermore, wounding causes a rapid, transient increase in jasmonic acid in tomato leaf cells (11). This implies that all of the enzymes necessary for converting linolenic acid to jasmonic acid are present in leaf cells and that the pathway is activated by wounding. Several inhibitors—i.e., sodium *p*-chloromercuribenzenesulfonate (PCMBS), sodium diethyldithiocarbamate (DIECA), and sodium salicylate (SA), have been identified recently (11–15) that block the octadecanoid pathway at different steps and inhibit proteinase inhibitor synthesis in response to wounding, systemin, and oligosaccharides at both the protein and mRNA levels. The effects of these inhibitors are consistent with the hypothesis that the primary signaling molecules generated at wound or infection sites activate defensive genes via the octadecanoid pathway. The pathway appears to be positively and negatively modulated by the plant hormones abscisic acid (16) and indole acetic acid (17), respectively.

In this report, we summarize the activities of various elicitors and inhibitors of the octadecanoid signaling pathway that regulate the localized and distal synthesis of proteinase inhibitors in response to wounding and elicitors. We provide evidence that the proteinase inhibitor-inducing activity of plant-derived oligogalacturonides and fungal cell wall-derived chitosan oligomers, like systemin, is inhibited by DIECA and SA and that all three elicitors produce an increase in jasmonic acid levels in tomato leaves. The data further support a role for the octadecanoid pathway in the signaling events that activate the expression of defensive genes in response to both herbivore and pathogen attacks.

MATERIALS AND METHODS

Chemicals. SA, PCMBS, and DIECA were obtained from Sigma. Systemin (7), oligouronides [degree of polymerization (DP) $\approx$ 20] (5), and nitrous acid-treated chitosan (DP $\approx$ 4 through >6) (6, 18) were prepared as described previously.

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Abbreviations: PCMBS, sodium *p*-chloromercuribenzenesulfonate; DIECA, sodium diethyldithiocarbamate; SA, sodium salicylate.

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Plants and Bioassay. *Lycopersicon esculentum*, variety Castlemart, plants were grown to the two-leaf stage (12–15 days after planting) under 17-h days at 28°C with >300 milliensteins (mE)·m^{-2}·sec^{-1} of light and 7-h nights at 18°C. Transformed tomato plants (*Lycopersicon esculentum*, variety Better Boy) expressing a prosystemin cDNA in the antisense orientation have been described (19).

To assay inducer and inhibitor chemicals, plants were excised at the base of the stem and placed in small vials containing 90 μl of various concentrations of inducers and/or inhibitors dissolved in 15 mM sodium phosphate (pH 6.5). Plants supplied with DIECA were pretreated through their cut stems with 90 μl of either phosphate buffer or 1 mM DIECA in phosphate buffer as appropriate and then supplied with 90 μl of phosphate buffer containing the inducers with or without 1 mM DIECA as appropriate. SA and PCMBS were supplied at 1 mM in the 90-μl aliquots containing the inducers. After having taken up the solutions (about 40–60 min), all plants, except those treated with SA, were transferred to 20-ml glass vials containing distilled water. Plants treated with SA were transferred to 20-ml glass vials containing 1 mM SA. All plants were then placed in a sealed transparent acrylic box containing a CO_2 trap (20) and incubated for 24 hr under constant light at 300 mE·m^{-2}·sec^{-1} at 28°C. Juice was expressed from the epicotyl tissue of individual plants by using a small mortar and pestle and assayed for proteinase inhibitor content by a radial diffusion immunoassay (21, 22).

Quantification of Jasmonic Acid in Leaves of Tomato Plants. A competitive enzyme-linked immunoassay was employed to determine the levels of jasmonic acid (3*R*,7*R* plus 3*R*,7*S* isomers) in extracts of tomato leaves as described by Weiler *et al.* (23).

RESULTS AND DISCUSSION

The Octadecanoid Pathway. The general features of the octadecanoid pathway (8), activated by systemin (wounding) and oligouronides (pathogen attacks), and three known inhibitors PCMBS (12), DIECA (13), and SA (11, 14, 15) and their sites of action are shown in Fig. 1. In this pathway, systemin and oligouronides are proposed to interact with plant cells to activate a mechanism that rapidly increases the intracellular levels of jasmonic acid (9). The intracellular events are likely triggered by the activation of a lipase that releases linolenic acid from cell membranes, but the origin of the linolenic acid and the mechanism of its production are still unresolved. The conversion of linolenic acid to jasmonic acid leads to the transcriptional activation of expression of defensive genes, such as proteinase inhibitors (9, 10, 24) and polyphenol oxidase (PPO) (3). Little is known of the mechanism of gene activation in response to wounding, but analyses of the 5′ regulatory regions of genes that are activated by jasmonic acid have revealed the presence of consensus sequences that appear to be involved with gene regulation by this molecule (25, 26).

PCMBS, an inhibitor of active apoplastic phloem loading in plants (27), blocked the induction of proteinase inhibitors induced by wounding and by systemin. The inhibition was reversed by reducing agents such as cysteine, dithiothreitol, and glutathione (12). The transport of radioactively labeled systemin and sucrose out of wounded leaves was similarly blocked by PCMBS, suggesting that PCMBS was inhibiting the active transport of systemin (12). However, PCMBS did not block the induction of proteinase inhibitors induced by oligouronides, linolenic acid, or jasmonic acid, indicating that the site of PCMBS inhibition was between systemin and linolenic acid, nor did it inhibit the signaling pathway for oligouronides (see Fig. 1).

On the other hand, DIECA, an active oxidant, inhibited the proteinase inhibitor-inducing activity of both systemin and oligouronides as well as the inducing activity of linolenic acid (13). DIECA was shown to inhibit the octadecanoid pathway by efficiently converting 13-hydroperoxylinolenic acid, the product of lipoxygenase on linolenic acid, to 13-hydroxylinolenic acid, which is not a signaling intermediate, thereby shunting the pathway into a dead end. The proteinase-inducing activity of 12-oxyphytodienoic acid, the next pathway inter-

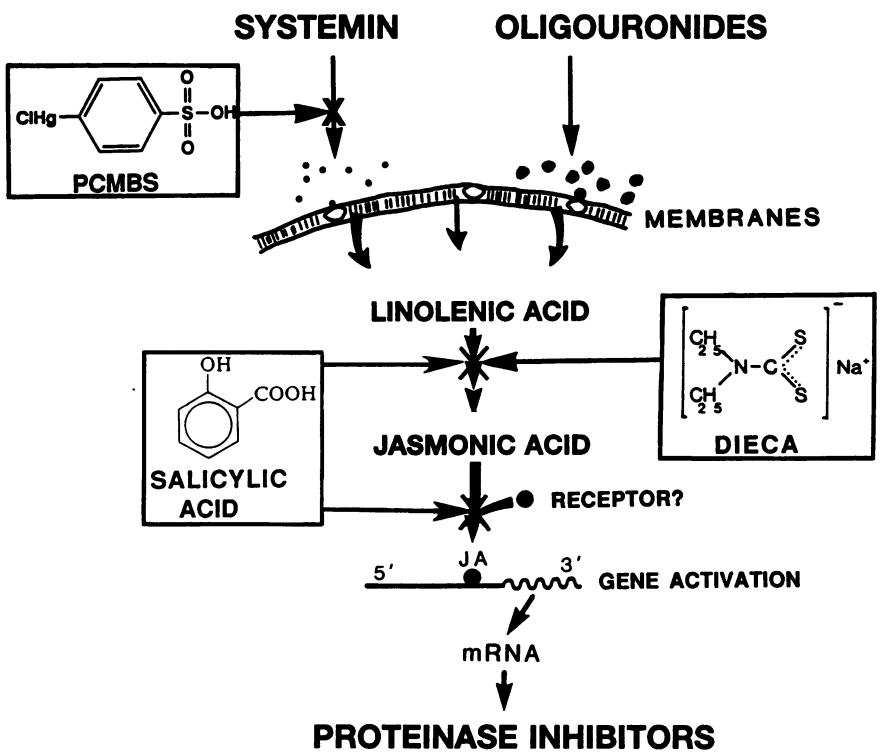

FIG. 1. Inhibitors (shown in boxes) of the octadecanoid signaling pathway for systemin and oligouronides, chemical signals generated at the sites of insect and pathogen attacks.

mediate downstream from 13-hydroperoxylinolenic acid, was not affected by DIECA (13). The experiments suggested that both systemin and oligouronides utilize the octadecanoid pathway but enter the pathway by different routes.

The inhibitory effect of SA on the synthesis of proteinase inhibitors induced by wounding, systemin, and oligouronides (14, 15, 16) also provided evidence that the three elicitors utilize the octadecanoid signaling pathway to signal defensive genes. SA is a key signal in the long-lasting systemic resistance in plants, called "systemic acquired resistance," that is induced by pathogen attacks (28). The array of defensive proteins induced by SA (28) differs from those induced by wounding, systemin, and oligouronides (3, 9, 10, 24), indicating that wound-inducible genes and SA-inducible genes utilize different signaling pathways. In 1988, SA was reported to be an inhibitor of the wound-induced and oligouronide-induced accumulation of proteinase inhibitors in tomato plants (15), but the cause of the inhibition was not known. More recently, the inhibitory activity was suggested to be a result of the inhibition of hydroperoxide dehydrase (11), thereby blocking the octadecanoid pathway and the synthesis of jasmonic acid. More recent experiments (14) have demonstrated that salicylic acid also inhibited proteinase inhibitor synthesis induced by jasmonic acid. The inhibitory activity of SA would effectively shut down the octadecanoid pathway in leaves in which SA levels are elevated in response to pathogen attacks. This suggested that the octadecanoid pathway and the systemic acquired resistance pathway can engage in a "cross talk" that can prioritize the pathways (14).

Chitosan oligomers, derived from fungal cell walls, are potent elicitors of plant defense responses (6, 18). On a weight basis, chitosan fragments are at least 10 times more potent in inducing proteinase inhibitors than oligogalacturonide fragments (18). Whether chitosans signal the induction of proteinase inhibitor synthesis through the octadecanoid pathway or through a separate pathway has not been investigated previously. In Table 1, the inducing activity of chitosan in young excised tomato plants is shown when supplied to young tomato plants in the presence and absence of the three octadecanoid pathway inhibitors shown in Fig. 1. Unaffected by PCMBS but strongly inhibited by DIECA and SA, chitosan inducing activity was similar to the signaling activity of oligouronides. This indicates that the pathway for the activation of proteinase inhibitor genes by chitosan likely involves the octadecanoid pathway.

Effects of Wounding, Systemin, and Oligosaccharides on Jasmonic Acid Levels in Leaves of Young Tomato Plants. Wounding of young tomato plants by crushing across terminal leaflets with a hemostat caused a 6-fold increase in jasmonic acid in the leaves within the first 2 hr after wounding (Fig. 2), then the levels decreased over the next several hours. This time frame is consistent with the timing of transient changes in proteinase inhibitor mRNA and protein synthesis in response to wounding (29).

Table 1. Effects of PCMBS, DIECA, and SA on the induction of proteinase inhibitor I accumulation in leaves of young tomato plants in response to chitosan

Treatment*	Inhibitor I accumulation, μg/ml leaf juice
Control (excised)	26 ± 18
Chitosan (5 μg per plant)	67 ± 33
+ PCMBS (1 mM)	69 ± 11
+ DIECA (1 mM)	31 ± 16
+ SA (1 mM)	30 ± 19

*Two-week-old tomato plants were excised at the base of the stem and supplied with chitosan for 30 min and then water or inhibitor solutions for 24 hr under constant light. Each value is the mean from six assays.

The levels of jasmonic acid in young excised tomato plants supplied with systemin, oligouronides, and chitosan were compared with levels in wounded plants 2 hr after wounding or treatment with elicitors (Table 2). Levels of jasmonic acid increased in response to all three elicitors and were at least 2 times higher than levels induced by wounding and were 10 to 15 times higher than those found in unwounded control plants. The results of these experiments, together with the effects of the various inhibitors presented above, strongly support a role for the octadecanoid pathway in the intracellular signaling of defensive genes by systemin, oligogalacturonides, and chitosan. The pathway likely activates several genes known to be induced in tomato leaves by jasmonates, including polyphenol oxidase (3), a sulfhydryl proteinase inhibitor (24), an acid proteinase (24), threonine deaminase (24), and aminopeptidase (24, 30).

Receptors for the three elicitors have not yet been identified, but it is possible, if not likely, that each of the different elicitors have different recognition processes that converge on a common pathway in which linolenic acid is released from cell membranes and converted to jasmonic acid. Oligouronides and chitin fragments have been shown to interact with tomato and potato plasma membranes to cause the phosphorylation of specific plasma membrane proteins (31, 32) and ion fluxes (32, 33), but protein phosphorylation events have not been reported that are associated with either systemin or chitosan signaling. A tomato plasma membrane protein of ≈50 kDa has been identified (34) that binds tightly to systemin and has properties similar to the furin class of processing enzymes found in animals and yeast (35), but no phosphorylation events have been associated with the enzyme, nor has a functional role been associated with its proteolytic activity.

While oligouronides and chitosan are commonly found in plants and fungi, respectively, systemin has been found only in solanaceous plants (7). Whether systemin is present in other plant families and whether other polypeptide signals are present in plants remain to be determined. The model in Fig. 1, which now includes chitosan as an extracellular signal, provides a framework to begin to understand the biochemistry of the signaling networks in plants that regulate the expression of defensive genes and perhaps the expression of environmental and developmental genes as well. It is becoming clear that the complexities of signaling systems of plants rival those of animals and that intensive research efforts will be required

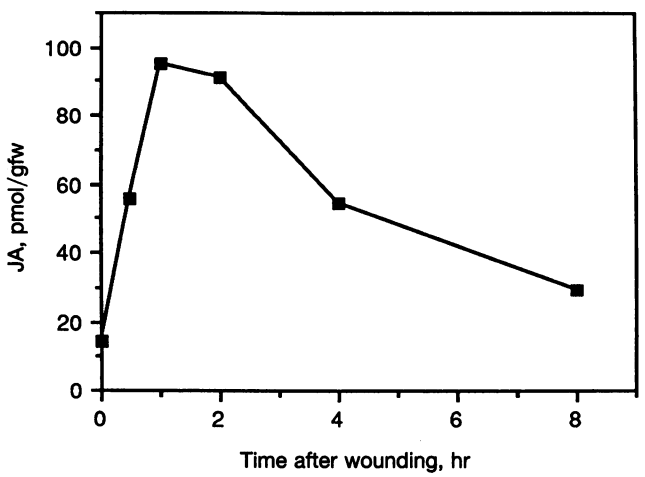

FIG. 2. The time course of the increase in jasmonic acid (JA) levels in leaves of young tomato plants that were wounded across the main veins with a hemostat to initiate the experiments. Each point represents the average from a set of 12 tomato plants. gfw, Gram(s) fresh weight.

Table 2. Increases in intracellular levels of jasmonic acid in leaves of young tomato plants in response to wounding and elicitors

Exp.	Treatment*	Jasmonic acid, pmol/g of tissue
1	Control (intact)	51 ± 8
	Wounded	136 ± 6
2	Control (excised)	30 ± 3
	Systemin (2.5 pmol per plant)	334 ± 16
3	Control (excised)	83 ± 31
	Oligouronides (200 μg per plant)	210 ± 67
4	Control (excised)	100 ± 13
	Chitosan (5 μg per plant)	332 ± 60

*Two-week-old tomato plants were either wounded across the main vein with a hemostat or excised at the base of the stem and supplied with elicitors for 30 min and then water for 2 hr under constant light. Each value represents the mean of three assays.

before a comprehensive understanding of these pathways in plants will be achieved.

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